UNIVERSITY OF PENNSYLVANIA

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A THESIS

IN PHYSIOLOGY

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THE EFFECT OF FATTY ACID BUFFER SYSTEMS ON THE APPARENT VISCOSITY OF THE *ARBACIA* EGG, WITH ESPECIAL REFERENCE TO THE QUESTION OF CELL PERMEABILITY TO IONS

EVELYN HOWARD[1]

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The subject of this study is the degree to which certain intracellular effects of acids may be modified by the presence of the salts of the acids. If a living cell is exposed to a penetrating acid and its salt, conditions being such that the salt is unable to penetrate, the internal reaction of the cell will be influenced by the absolute concentration of the acid rather than by the pH of the solution as such. Results which were interpreted in this way, as evidence of the relative impermeability of cells to salts, have been obtained for a variety of cells by Jacobs (1920*a*, 1920*b*, 1922*a*), Beerman (1924), Bodine (1925), and Lillie (1926, 1927). Furthermore, Osterhout (1925) and Osterhout and Dorcas (1926) have investigated the penetration of CO_2 and H_2S in a more quantitative manner on a particularly favorable material, *Valonia*, with results which led them to state that ". . . it is only the undissociated molecules which penetrate. . . . Under ordinary conditions there seems to be little or no exchange of ions."

On the other hand Smith and Clowes (1924*a*, *b*), and Smith (1925, 1926) found that certain physiological effects of the free acid in buffer systems of penetrating acids depended on the pH of the external solution, a result which they were inclined to interpret (1924*b*) as being due to the penetration of the salt of the acid rather than to effects on the external cell surface, since analogous external pH variations produced by non-penetrating acids were physiologically ineffective. Similar conclusions as to the penetration of certain salts have been reported by M. M. Brooks (1923) and by Haywood (1927).

The contradiction implied in the conclusions of these two groups of workers led to the present study of the question, in which the effect of acids on the apparent viscosity of the protoplasm of sea urchin eggs has been used as a measure of the intracellular effectiveness of the pH produced by various systems. The results obtained indicate that physi-

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ologically significant quantities of the salts of several penetrating acids enter the cells studied within a few minutes, in contrast to the ions of certain mineral acids, for which there was found no evidence of penetration under similar conditions. In this respect my work confirms the conclusions of Smith, but it has extended them to apply to considerably shorter times, and has led to a different conception of the manner in which the intracellular buffering action of the salt might be brought about.

Material and Methods

The unfertilized eggs of the sea urchin, *Arbacia punctulata,* have been used throughout these experiments, since they are adaptable to semi-quantitative studies on the apparent viscosity of protoplasm. Because of the danger that the selective permeability of cells might be altered by injury, care was taken that the material should be as normal as possible. Eggs which were in good condition, as indicated by the prompt formation of fertilization membranes and by uniform cleavage (95 to 100 per cent), stood centrifuging without appreciable cytolysis and as a rule the controls maintained a uniform value of the apparent viscosity throughout an experiment. During the beginning and the end of the season, a sample of eggs of each lot studied was fertilized, and those lots selected for use which showed prompt and uniform elevation of fertilization membranes, but in the middle of the season such a selection was unnecessary. The occurrence of an apparent liquefaction on treatment with acid is not incompatible with subsequent cleavage and development after fertilization on return to sea water, although progressively less recovery is obtained as the degree of liquefaction is increased.

An experiment consisted of exposing the cells to the solutions in question, and determining the apparent viscosity of the protoplasm after various intervals. The method of centrifugation employed for these measurements was composed essentially of observations on the degree of movement of the yolk granules through the cell under the influence of a given centrifugal force acting for a constant time. Certain factors concerned in the resultant apparent viscosity of the protoplasm of these cells will be discussed elsewhere. For the present purposes such a determination of the rate of granule displacement has been used as a measure of the effects of acid, without attempting any analysis of the actual changes occurring in the apparent viscosity, or of whether changes occurred in the granules themselves. The term liquefaction is used in this paper to describe the effects studied, because it is probable from the results of various authors (Jacobs, 1922*b*; Edwards, 1923; and

Brinley, 1928) that at least a part of the physical effects of acid on the cell consist in the production of an actual liquefaction.

The granule displacement was evaluated in the following manner. After centrifuging, in order to prevent return redistribution of the granules in the cell, the eggs were immediately fixed in a solution of 0.04 per cent formaldehyde in sea water. The layers of material which are separated in sea urchin eggs by centrifugal force may be classified as a cap of fatty material, a hyalin segment of clear protoplasm, a segment of yolk granules, and finally the red pigment granules. Counts were made of the percentage of eggs showing a hyalin segment of an altitude of one-fifth or over of the diameter of the egg, out of a total number of 200 eggs which showed an axis of stratification perpendicular to the axis of observation. The position of the axis of stratification can be determined in a definite manner by locating the red granule layer opposite to the clear area. Since the estimation of the position of this axis is only possible in those eggs which show some granule displacement, the percentage actually counted is not an absolute percentage representative of the entire lot of eggs, although it tends to become so as its magnitude increases. The altitude of the hyalin segment was measured from the surface of the egg to the boundary between the hyalin and yolk segments, including within this segment the small amount of fatty material. The measurement consisted simply of a comparison with an ocular micrometer scale at a magnification of 100 diameters. The counts were reproducible with a probable error of plus or minus one per cent. The percentage of eggs showing this degree of stratification as determined in the manner described will hereafter be referred to as sigma.

Counts made on the same samples of eggs at intervals after the fixation showed an initial increase of about eight sigma during the first twenty minutes following fixation, after which the value remained constant for about two hours. A slow decrease in sigma of the order of 6 to 8 occurred after four to eight hours. Slightly lower concentrations of fixative did not give the initial rise but gave a more rapid drop, and higher concentrations tended to render the cells opaque. With the concentration of fixative employed, counts were made between one-half hour to two hours after fixation.

For values between 5 and 40, sigma increases nearly linearly with the time of centrifuging; but below 5 the curve of sigma against time is convex to the time axis, and above 40 to 60 sigma the curve tends to become concave. Treatment with fatty acids causes sigma to increase until a point is reached, with increasing concentrations of acid, where coagulation begins to occur, which is associated with an abnormal

general appearance of the eggs. Coagulating concentrations of acid were not used in the present study of permeability.

Numerical values of sigma in different acid concentrations are of relative significance only, since considerable variations in absolute magnitude occurred among different lots of eggs. In the experiments described below, however, the relative effectiveness of a series of solutions was reproducible without exceptions. The effects described are based on a series of observations on three or more lots of eggs, for each series of solutions tested.

A centrifugal force of approximately 1000 times gravity was used, but this was not entirely constant from day to day, since the line current at Woods Hole fluctuates considerably. However, controls in sea water or unbuffered saline were included in each test, and with material in good condition sigma values were usually constant during each experiment, within the limits of error of the counts. The centrifuge was accelerated in a standard manner, twenty-five seconds being used to move the rheostat to its final point, and the machine established speed in about forty seconds after the current was turned on. Room temperatures varied between 22 and 25° C. during the course of the work, but the variations during any one experiment were not more than a few tenths of a degree. The cells were centrifuged for 2.0 to 2.5 minutes, the actual time suitable for each lot being determined in a preliminary test.

The technique of measuring the apparent viscosity differs from that previously employed by Heilbrunn (for numerous references see 1927) and by Barth (1929) in one fairly important respect. These workers, by centrifuging the material for various times, determined the shortest time required to produce granule displacement for comparison under different conditions. For the present purposes, I found that in cells centrifuged for any one time, with the technique used, the amount of granule displacement varied in different cells to such an extent that it was not possible to compare groups of cells centrifuged for different times unless comparisons were made on the basis of those times at which equivalent percentages of cells showed a given amount of granule displacement. In practice it was found more satisfactory to compare the percentages of cells showing a given amount of granule displacement after equal times of centrifuging.

Solutions.—Stock solutions of acids were made up by titration against standard NaOH, using phenolphthalein as indicator, dilution to the desired concentration, and retitration. The solutions of the salts of the fatty acids were made up by mixing 1.000 N NaOH and 1.000 N acid to a pH of 9.4 with thymol blue, this being the approximate pH of salt hydrolysis, allowing for salt error of the indicator, at salt

concentrations of 0.5 molar. The salt solutions were kept on ice and made up fresh each week. Solutions for use on the eggs were made up daily from these stock solutions by volumetric dilution, with an accuracy of 0.5 per cent, in balanced isotonic saline media. For the first part of the work the saline medium was prepared daily from sea water, by treating it with 5 cc. of 0.5 N HCl per liter and passing a current of moist room air through it overnight. This procedure frees the solution from carbonates and CO_2 except for traces in equilibrium with room air. The solution was then filtered and neutralized with 0.25 N NaOH to an apparent pH of 7.2 with phenol red, which, corrected for salt error, becomes pH 7.0. To this medium were then added the acids to be tested on the eggs. However, for most of the work an artificial saline was prepared daily by mixing 1.00 M NaCl 420 cc., 0.50 M KCl 18 cc., 0.50 M $MgSO_4$ 51 cc., 0.50 M $MgCl_2$ 46.7 cc., and 0.50 M $CaCl_2$ 18.7 cc., and distilled water to one liter. These concentrations of salts were obtained from data on the relative salt concentrations of sea water, as given in the Tabulæ Biologicæ. The salinity was adjusted to the point where the medium caused no significant change in sigma, when compared with Woods Hole sea water. No difference in the results was observed between solutions made up in this saline and in the carbonate-free sea water.

When the volume of hypotonic reagents added to the saline exceeded one per cent, hypertonic NaCl was added in proper amount to restore the osmotic balance. Two per cent of distilled water in sea water was found not to affect sigma, while 10 per cent distinctly increased sigma. When the volume of the solutions added to the saline exceeded 10 per cent, the amounts of K, Ca, and Mg were adjusted to maintain the normal cation ratio, unless otherwise stated. A ten per cent variation in the cation ratio was not associated with appreciable variations in sigma. The effect of larger variations is described below.

All pH values given for HCl and phosphate solutions were determined with a quinhydrone electrode. The individual determinations were subject to potential drifts of one to three millivolts. The values given in Table I are averages of several determinations. During preliminary experiments, the pH of the solutions was found to remain unchanged during an experiment. The pH of solutions of fatty acid buffers were calculated from the known amounts of the free acid and its salt added, using constants of pK′ as 4.48 for acetic acid and 4.56 for valeric. The constant for acetic was taken from data of Michaelis and Krüger (1921) as the value of the constant in 0.5 M NaCl with 1/50 M $CaCl_2$, and the value for valeric was calculated from the accepted value for its pK, assuming that the effect of the salinity is the

same as with acetic acid. For the second dissociation constant of phosphoric acid Michaelis and Krüger's value of pK′ in 0.5 M NaCl of 6.43 was used.

It will be noticed that the procedure followed in making up the experimental solutions differed from that used by Smith in his work on marine ova in one fairly important respect. Smith's method was to add standard sodium acetate to neutral carbonate-free sea water, and then to divide the resulting solutions into separate portions, and adjust the hydrogen ion concentrations to various values by the addition of HCl of the appropriate strength. This technique obviously lowers the salt concentration at the same time that it raises that of the free fatty acid, thus changing simultaneously the three variables: salt, acid, and pH. Under these circumstances the evaluation of the effect of each variable separately is difficult, and can be attempted only by a combination of a number of experiments. My procedure was so planned that each of the variables, acid, salt, and pH was in turn held constant while the other two were varied, thus rendering it possible to test Smith's conclusions directly in individual experiments.

The Effect of Certain Acids and Buffer Systems on the Apparent Viscosity

1. *The Effect of Mineral Acids on the Apparent Viscosity*

One of the arguments that ions in general do not penetrate living cells has been that cells are not injured or affected by the completely dissociated strong acids or bases in pH ranges in which the solutions of certain weak acids and bases, which are believed to contain a large proportion of undissociated molecules, can exert marked physiological effects apparently resulting from their ability to affect the hydrogen ion equilibria within the cell. The work of Bethe (1909), Warburg (1910), Harvey (1911), Jacobs (1920*b*), Chambers (1928) and others has shown that weak acids and bases produce intracellular indicator changes promptly under conditions where strong acids and bases are ineffective. A variety of physiological effects in animal, plant, and bacterial cells have been shown to follow the same rule in that, according to Loeb (1909), Cohen and Clark (1919), Jacobs (1924), Smith and Clowes (1924 *a* and *b*), Barth (1929) and others, they respond to weak organic acids more readily than to strong mineral acids, although in application the rule is not without its limiting conditions, especially when high concentrations of acids are employed (Crozier, 1916).

In the present experiments this general principle has been confirmed, in that HCl and the phosphate buffer system have been found to be

ineffective in producing a liquefaction of protoplasm within the pH range in which the organic acids were effective.

Eggs were exposed to acids for various times, and they were then centrifuged and sigma values determined in the manner described above. The results of exposing eggs to HCl in unbuffered saline for fifteen minutes are given in Table I.

TABLE I

The Effect of HCl on the Apparent Viscosity

Experiment	Molarity	pH observed	Sigma
1	1.1×10^{-5}	—	15
	2.2	5.42	12
	4.3	5.08	16
	control	—	12
2	4.3	5.08	17
	control	—	17
3	5.4	4.41	12
	6.5	4.34	18.5
	8.7	4.28	15.5
	control	—	14.5

Since the variations of sigma in cells in HCl solutions are slight, and since there is no regular variation with the changes in acid concentration, it is considered that no significant effects on the apparent viscosity are produced by HCl at pH 4.3 or above. At about pH 4.3 the eggs tend to cytolyze, so that in view of complications due to cell injury, quantitative measurements of sigma below pH 4.3 were not undertaken. At pH 4.1 the protoplasm was not coagulated after fifteen minutes' exposure, and at pH 3.0 coagulation occurred.

Similar experiments were performed with phosphate buffers. Solutions of 0.00132 and 0.0066 molar NaH_2PO_4 in saline media were adjusted to a series of pH values between 4.2 and 5.8 with NaOH. No significant differences were observed between the apparent viscosity of cells in the saline media alone and in these phosphate solutions, after exposures of five to forty minutes. The ions of the phosphate system therefore appear to resemble the ions of HCl in being unable to influence the interior of the living *Arbacia* egg. The pH ranges used with these mineral acids are within those in which the fatty acids exert their effects; consequently the effects produced by the fatty acids cannot be due to the hydrogen ion concentrations as such which are produced in the external media.

2. *The Effect of Fatty Acids on the Apparent Viscosity*

The results obtained with the saturated fatty acids are very different from those just described for mineral acids. The effect of unbuffered valeric acid on protoplasmic viscosity is shown in Fig. 1. In this and all subsequent figures the ordinates represent the degree of liquefaction in sigma, and the abscissæ the time in minutes of exposure of the cells to the various solutions up to the time of centrifuging. In Fig. 1 it will be observed that with increasing time of exposure the degree of liquefaction increases up to a certain time, beyond which it tends to decrease. The magnitude of the maximum liquefaction and the rate of change increase with the concentration of the acid, between 1×10^{-4} and 3×10^{-6} molar.

Since the action of valeric acid may be due in part to the liberation of carbon dioxide from intracellular carbonates, a few observations on the effect of carbon dioxide were made. The gas was passed through sea water until a saturated solution was obtained. The latter was then diluted with sea water to certain pH values, determined with the quinhydrone electrode. A marked liquefaction was obtained at pH 5.1, and a coagulation at pH 4.9. These pH values correspond to carbon dioxide tensions of approximately 400 and 700 mm. respectively, according to the data of Henderson and Cohn (1916).

The magnitude of the liquefaction obtainable with unbuffered valeric acid is not very great. Cytolytic effects usually begin to occur above concentrations of 0.0001 molar, obscuring the possible effects of larger concentrations of acid on protoplasmic viscosity. Out of a total of seven experiments with unbuffered valeric acid, a liquefaction was observed six times; and in the lot of eggs in which no definite liquefaction could be observed, appreciable cytolysis was produced by 0.0001 N acid.

Using acetic acid in unbuffered solutions, I failed to observe a liquefaction in four experiments, using concentrations of 1×10^{-4} to 1×10^{-6} normal, although considerable cytolysis was observed in the higher concentrations. Consequently it is obvious that acetic acid produces intracellular effects less readily than valeric.

The effectiveness of different fatty acids was compared in buffered solutions, since under these conditions it was possible to obtain a definite liquefaction without cytolysis in all the acids used. The effect of the length of the carbon chain was studied with the acids acetic, propionic, butyric (a mixture of normal and iso forms), and *n*-valeric. Because of the almost equal strengths of the acids in question, such a comparison has a greater significance than would otherwise be the case. The results of a typical experiment with short exposures are shown in Fig. 2.

The concentration of the free acid was in each case 0.000517 N, in the presence of 0.000993 mols per liter of its salt. In this experiment the salt concentration was determined from the amount of standard NaOH added to the respective standardized acid solutions, instead of the salt having been made up separately as described above for the other experiments. The following pH values were calculated for these solutions, using the dissociation constants obtained by Drucker (1905) and assuming the correction of —.249 applied to pK for acetic acid to hold for each acid: acetate system 4.77, propionate 4.87, butyrate 4.81, and valerate 4.84. It is evident from Fig. 2, that the rapidity with which liquefaction is produced by such buffer mixtures increases with the increasing length of the carbon chain, irrespective of the slight differences in pH, which, except in the case of the propionic acid, would

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PLATE I

Explanation of Figures

The figures show the effect of various acid solutions on the apparent viscosity of the *Arbacia* egg. The ordinates are the values of the viscosity in sigma, plotted in each case to the scale shown in Fig. 1, although only relative viscosities rather than absolute values are comparable in separate figures. The abscissæ are the times of exposure of the eggs to the solutions, up to the times of starting the centrifuging. The diagrams give the time in minutes, and it will be noticed that the time scales are not the same in all the figures. The curves are numbered in the order of increasing hydrogen ion concentration. The solutions as given were made up in balanced isotonic saline. The effects shown in Figs. 3 to 6 are typical of both acetic and valeric buffer systems.

Curve 1 in each figure represents the controls in sea water or in the unbuffered saline medium.

1. The effect of unbuffered solutions of valeric acid. Curve 2, valeric acid $2.9 \cdot 10^{-6}$ normal; curve 3, $1.74 \cdot 10^{-5}$ normal, curve 4, $1.16 \cdot 10^{-4}$ normal.

2. The effect of the length of the fatty acid carbon chain on the rapidity with which the liquefaction is produced. Curve *A*, acetic acid; curve *P*, propionic acid; curve *B*, butyric acid; curve *V*, valeric acid. The acid was in each case $5.17 \cdot 10^{-4}$ normal, in the presence of $9.93 \cdot 10^{-4}$ mols of the respective salt.

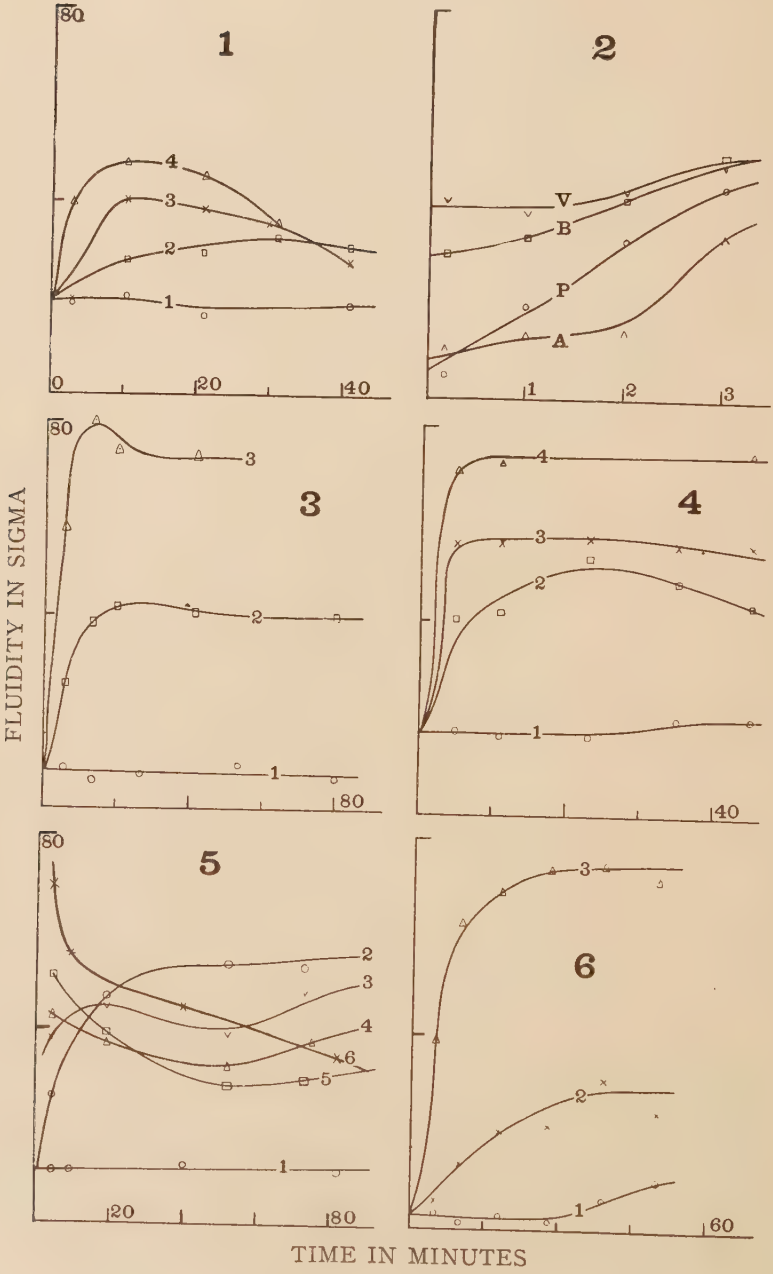
3. The effect of varying the pH by varying the concentration of the free acid in the presence of a constant amount of the salt of the acid. Curve 2, pH 5.2, free valeric acid 0.000341 normal; curve 3, pH 4.8, acid 0.000852 molar; sodium valerate in each case 0.00135 molar.

4. The effect of varying the pH by varying the concentration of the salt of the acid in the presence of a constant amount of free acid. Free valeric acid, 0.000585 normal. Curve 2, pH 5.6, sodium valerate 0.00585 molar; Curve 3, pH 5.0, valerate 0.00176 molar; curve 4, pH 4.6, valerate 0.000585 molar.

5. The effect of varying the salt of the acid in the presence of a constant amount of acid at a higher pH range than that of Fig. 4, namely 5.4 to 6.7. Free acetic acid 0.00096 normal, in the presence of sodium acetate of 0.008 to 0.16 molar. Curve 2, pH 6.7; curve 3, 6.4; curve 4, 6.0; curve 5, 5.7; and curve 6, 5.4.

6. The effect of increasing both the acid and the salt, while maintaining a constant pH of 5.17. Curve 2, free valeric acid 0.00029, valerate 0.00117; curve 3, acid 0.000870, valerate 0.00351 molar.

PLATE I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FLUIDITY IN SIGMA
TIME IN MINUTES

be expected to reduce rather than to enhance the observed effects of the permeability differences.

After three to five minutes' exposure to the fatty acid solutions, there is shown a tendency of the viscosity values to reach the same equilibrium point. However, an identical equilibrium is not realized, and even after twenty minutes' exposure the acetic acid has usually produced somewhat less liquefaction than the valeric, in spite of the fact that it is a slightly stronger acid, this behavior recalling the comparative ineffectiveness of acetic acid in unbuffered solutions.

The observation that the rate of production of the protoplasmic liquefaction increases with the length of the fatty acid carbon chain accords with the general principle of Overton, which seems to hold for a great variety of material (Jacobs, 1924), that in homologous series, the more highly the non-polar portion of a molecule is developed as compared with the polar, the more readily are intracellular effects produced. The ineffectiveness of the mineral acids in this case cannot be due to their low concentrations, for HCl fails to produce a liquefaction at a concentration of 8.7×10^{-5}, while valeric acid is effective at 3×10^{-6} N. Furthermore, if we consider the buffering capacity of the solutions used ($\Delta B/\Delta pH$, Van Slyke, 1922) over the pH interval of 4.77 to 6.80, the latter taken as the normal pH of the cell contents (as determined for other *Echinoderm* ova by Chambers, 1928), it is seen that the effective acetate solution has a buffering capacity of only 0.00025, while for the ineffective phosphate buffer it is 0.0023.

3. *Penetration of the Cell by the Salts of Fatty Acids*

So far the results have not been in disagreement with the conceptions that ions penetrate uninjured cells only with considerable difficulty, if at all. If now we consider the salts of the fatty acids, which are believed to be completely ionized, we obtain quite a different picture. For the following experiments in this section both acetate and valerate buffers have been used.

(a) *The Effect of the Salt of the Fatty Acid on the Apparent Viscosity*

As a preliminary to the study of the effect of the acid in the presence of the salt, observations were made on the effect of the salt alone. Sodium acetate in the saline media without the presence of free acid, other than the traces resulting from hydrolysis, was found to produce no significant changes in sigma in concentrations of 0.001 to 0.004 molar. In solutions of 0.008 and 0.01 molar, a liquefaction was observed after twenty minutes' exposure which became more marked after

longer exposures. The effect was variable in degree, but the liquefaction was at times as great as thirty sigma above the controls after forty minutes' exposure to 0.01 molar sodium acetate. In the presence of a potassium excess of 0.01 molar, the acetate effect was increased, *i.e.,* it was manifested sooner and in greater degree. Lithium acetate, 0.01 molar, produced an even greater effect than potassium acetate. The lithium and extra potassium, when added in the form of chlorides to the standard saline, caused no significant changes in sigma; therefore, this liquefying effect of lithium or of excess potassium appears to be associated with the presence of the acetate ion.

(b) *The Effect on the Apparent Viscosity of Changing the pH by Varying the Free Acid in the Presence of Constant Salt*

In a concentration range in which the salt alone produced no detectable effects, it may be shown that the effect of the acid on the apparent viscosity in the presence of the salt is similar to the effect of the acid in the absence of the salt in the case of valeric acid described above. The result of varying the free acid in the presence of a constant amount of salt is shown in Fig. 3. It is seen that, in the presence of the salt, as with the acid alone, increasing degrees of liquefaction are associated with increasing concentrations of free acid. This result would be expected whether or not the salt penetrated. The concentrations of acid used are greater than those possible in unbuffered solutions without gross injury; and the liquefaction produced is also greater and considerably more prolonged.

(c) *The Effect on the Apparent Viscosity of Changing the pH by Varying the Salt while the Free Acid is Constant*

If the salt penetrates readily, it would be expected that altering the pH by varying the salt concentration in the presence of a constant amount of free acid would result in immediate differences in the degree of liquefaction produced by the acid. That such is the case is shown in Fig. 4, in which the effect of valeric acid 0.000585 N is shown to be modified by sodium valerate of 0.00585 to 0.000585 molar. It is seen in Fig. 4 that the degree of liquefaction increases with the increasing hydrogen ion concentration and the decreasing amounts of salt. At pH 4.6 to 5.6 this effect is marked in both the valeric and the acetic solutions. The effect of the salt appears within four minutes of exposure to the solutions, the shortest time tested. It is presumably not a specific salt effect, since it occurs in salt concentrations of 0.002 to 0.0006 molar, which are below those where the salt effect was ob-

served in solutions of sodium acetate without added free acid, and also because the liquefaction increases rather than decreases with the decreasing salt concentration. It is probably not caused by an influence of the salt on the rate of penetration of the acid, since the acid penetrates very quickly and apparently establishes a distribution equilibrium within three to eight minutes in solutions of pH 5.2 or below.

It seems most probable that the influence of the salt is due to an actual buffering effect exerted intracellularly. If this is so, these experiments indicate that the effect of the acid on the apparent viscosity is exerted through some influence on the intracellular hydrogen ion equilibria, rather than through a more specific molecular reaction, since, when the total acid concentration is constant, variations in the hydrogen ion concentration alter the effectiveness of the acid. It is believed, therefore, that these experiments constitute evidence that the salts of fatty acids are able to penetrate the living cell. In regard to the comparative rates of penetration of the acids and their salts, it is shown in Fig. 2 that acetic acid does not exert its characteristic effect until after about three minutes' exposure. Since the buffering action of sodium acetate may be in evidence after four minutes' exposure (the shortest time tested), it appears quite possible that the salt penetrates the cell, in the presence of the acid, at a rate which is of the same order of magnitude as that of the acid.

When a similar experiment is performed in which the free acid is kept constant and the salt varied, within a higher pH range, *i.e.*, between 5.7 and 6.7, somewhat different results are obtained. In order to produce a measurable liquefaction at these pH values, both the acid and the salt must be increased to within the range of salt concentrations at which the salt produces specific effects on the apparent viscosity. The results of such an experiment are shown in Fig. 5. The free acid in this experiment was 9.6×10^{-4} N, and the salt concentrations were up to 0.16 molar. The pH values calculated were, for curve 2, 6.7; curve 3, 6.4; curve 4, 6.0; for curve 5, 5.7; and curve 6, 5.4. It is seen that the initial effect is a liquefaction which increases with the hydrogen ion concentration, as was observed for the lower pH ranges described above. However, with increasing exposures, sigma values decrease in the solutions of lower pH and rise in the higher, with the result that, after an hour or more of exposure, between pH 5.7 and 6.7 the order of increasing fluidity has become reversed and consequently the liquefaction increases with the salt concentration instead of with the external hydrogen ion concentration.

Since the order of increasing fluidity may be reversed in this way, it is possible that the liquefying action of the salt may be of a different

character from that of the acid. In view of the many factors which may affect the apparent viscosity, it is not unlikely that the seat of action of the hydrogen ion may differ from that of the salt. In this connection, the possibility was considered that the influence of the salt on the apparent viscosity was the result of an osmotic effect. The solutions of sodium acetate in saline media were made up to be isosmotic with normal saline, but if the salt should penetrate and remain osmotically active, these solutions would not be isotonic for the cell; in fact, the solutions used in the experiment shown in Fig. 5 would in effect be of various degrees of hypotonicity down to 68 per cent of the isotonic strength, and the expected changes in volume would result in an increased apparent liquefaction such as was observed. That this is probably not the case, however, is indicated by the fact that measurements of cell volumes in such solutions, kindly made for me by Miss D. R. Stewart by a technique which she will describe elsewhere,[2] revealed no significant changes. In this respect, then, the sodium salts of the fatty acids differ from the ammonium salts, which Stewart (1929) has found to cause osmotic swelling. These results may be due to the fact that the sodium salts can enter only in osmotically negligible amounts, but, on the other hand, it may be that their entrance is for some other reason not associated with an increase in the total osmotic pressure of the cell.

(d) *The Effect on the Apparent Viscosity of Increasing the Total Acid at Constant pH*

When the pH is kept constant and the free acid and the salt increased simultaneously a greater liquefaction is produced as the free acid concentration becomes larger, as shown in Fig. 6. Indeed, a coagulation was obtained with free acid above 0.001 N at pH 5.2. This result might be due to the fact that relatively less salt than acid penetrates the cell, so that the intracellular hydrogen ion concentration would become greater with the larger amount of acid. On the other hand there is an alternative possibility that the buffering capacity of the penetrating acid solution is one of the factors concerned in the magnitude of the changes produced. That buffering capacity is of importance in these experiments may be inferred from the work of Chambers (1928), Reznikoff and Pollack (1928) and Pollack (1928), who find that various cells have considerable buffering power, sufficient indeed to prevent changes in the colorimetrically determined pH of the hyalin protoplasm, within the observational limits of $\pm$ 0.1 pH, when treated with sublethal concentrations of CO_2. Hence it would be expected that

[2] See Stewart, *Biol. Bull,* in press.

the production of an acid liquefaction in the present experiments is not associated with an equalization of the intracellular and environmental pH, but only with a certain shift of the pH of the cell. The degree of this shift would be a function not only of the pH itself, but also of the buffering capacity of the penetrating components of the medium. Consequently, on this basis, one would expect the results obtained in this experiment, and such effects cannot be considered as evidence that the salt penetrates less readily than the acid.

A Possible Mechanism by which the Salts of Penetrating Acids Might Enter the Cell

The apparent permeability of the cell to the salts of the penetrating fatty acids presents a rather surprising contrast to its relative impermeability to certain other ionized compounds. This situation might be due to specific differences in the behavior of the different ions toward the cell membrane, but there is no independent evidence that the physical properties of the salts of the various acids are related to the physical properties of the acid molecules to an extent which would account for these physiological peculiarities.

An alternative explanation may be developed from the following considerations. In general, ionized compounds do not penetrate cells at all freely, but it is possible that the restrictions to the passage of ions in many cases are imposed chiefly on ions of one sign. The erythrocyte, for example, under ordinary physiological conditions, appears to be a cell which is permeable to anions and impermeable to cations (for references see Warburg, 1922 and Van Slyke, Wu, and McLean, 1923). The fact that the erythrocyte is freely permeable to ammonium chloride and similar ammonium salts has been explained by Jacobs (1927) as being due to the free penetration of the cell by NH_3 and subsequent exchange of the internal OH ions for the external anions of the salt. With the majority of cells, it is possible that the conditions prevailing in the erythrocyte may be reversed, such cells under ordinary conditions being more permeable to cations than to anions. The evidence for other cells is less direct than in the case of the erythrocyte, and is chiefly potentiometric, the sign or magnitude of the potentials measured across membranes responding to changes in the concentration or character of the salt present in the manner which would be expected if the anions could not pass through the membrane while the cations tended to diffuse. The application of the principles involved has been discussed by Michaelis and others (1925, 1927, etc.). Potentiometric evidence of this nature has been reported by Loeb and Beutner (1911)

and by Fujita (1925) for the apple skin, by Mond (1927) for the wall of the stomach, by Amberson and Klein, (1928) for the frog skin, and by Sumwalt (1929) for the chorion of the *Fundulus* egg. Michaelis and Fujita (1925) have furnished in addition chemical evidence of the permeability to cations and the impermeability to anions of the apple skin. It is generally known that a normal surface is positive to an injured area in nerve, muscle, and apple, and this sign of the demarcation potential is not incompatible with a greater permeability of the normal surface to cations than to anions. Furthermore, Mond and Amson, (1928), from analyses of perfusing fluids, concluded that frog muscle is permeable to certain cations, *e.g.,* $K^{\cdot}$ and $Cs^{\cdot}$, but is impermeable to chlorides.

If the *Arbacia* egg is permeable to certain cations, its apparent permeability to the salts of penetrating acids might result from the entrance of the cell by the organic acid in the undissociated form followed by a transfer of cations, the external base being exchanged for the hydrogen ion derived from the intracellular dissociation of the acid. This process would result in the presence of acid anions and additional base inside the cell without an actual transfer of anions across the membrane. There is evidently an analogy between this mechanism and that of the penetration of the anion-permeable erythrocyte by a salt whose cation enters in an indirect manner. However, conditions are not completely analogous, since the penetration of the erythrocyte by ammonium chloride causes marked osmotic swelling, whereas this does not appear to be the case in the penetration of the *Arbacia* egg by potassium acetate.

If the transfer of base takes place by an exchange of alkali cations from without for hydrogen ions from within, the mobility of the cations in passing through the membrane might be the limiting factor in the rate at which the intracellular buffering action of the salt becomes manifest. Michaelis and collaborators (1925–1927) have observed that in the dried collodion membrane the mobility differences of the cations are greatly exaggerated. Netter (1928), Brooks (1930), and others have applied these considerations to explain the accumulation of K by many cells as a result of a cation exchange of hydrogen ions, produced metabolically, for K ions from the outside rather than for Na ions, since, although the latter ions are present in much the greater concentration in the environment, their diffusion may largely be restrained by a membrane which permits the passage of K ions. The presence of a membrane of this type in *Arbacia* eggs is suggested by the high ratio of K to Na found in the cells in comparison with that in sea water. Blanchard (unpublished results) reports a ratio of equivalents of K to Na of 1.90 in *Arbacia* eggs, while in sea water the ratio is 0.0213.

If this property of the cells results from characteristics of the membrane, it would be expected that, in the exposure of the cells to an acetate buffer in the balanced saline media, the base transferred would be K rather than Na, and that consequently changes in the K concentration of the medium would affect the rate of entrance of the base, and thereby the degree to which a given amount of salt is able to exert an intracellular buffering action after short exposures. This was observed to be the case under the following conditions. Certain amounts of 0.05 N NaCl, KCl, or RbCl were added to solutions of acetate buffers, with free acetic acid 0.00059 N at pH 5.0, in the usual saline media. As already mentioned, variations in K concentration of this magnitude in the absence of acetates produced no significant changes in sigma. The effects on the degree of liquefaction produced during the first two to nine minutes of exposure to such solutions are shown in Table II. The results are given in sigma reduced to a common basis by taking the values obtained in the high K concentration as twenty sigma.

TABLE II

The Effect of Various Cations on the Liquefaction Produced by an Acetate Buffer

Concentration of chlorides added *mols/liter*	Rb	K	Na
0.002	—	20	35
0.002	7.5	20	27.5
0.01	14	20	28
0.004	13.5	20	—
0.003	—	20	32

In these experiments the absolute differences are small, but the relative effects of the cations always appeared in the order given at this pH. At higher pH values, where the salt reversal of the pH effect occurred, the cation series was in most cases reversed also. However, if we consider only the pH range where the salt appears to exert a buffering effect which is not complicated by other factors, the degree of initial liquefaction produced is in the order Rb<K<Na. After longer exposures, there is a tendency for the same equilibrium point to be reached, but this is not maintained for over twenty minutes in some cases. It will be noted that the series obtained is compatible with the view that the degree to which the salt is able to buffer the acid intracellularly during the first few minutes of exposure, and therefore the rate of penetration of the salt, increases with the amount of the more highly mobile cations present. Although physical properties

other than mobility may be concerned, nevertheless the effect is suggestive of an actual penetration of base rather than a removal of acid as suggested by Smith.

When the amounts of acetate in the medium are as high as 0.1 molar, such a cation exchange would probably result in an abnormally great accumulation of K in the cell relative to Ca. Chambers and Reznikoff (1926) have observed that the injection of NaCl or KCl into cells causes a liquefaction, whereas Ca causes a coagulation. This observation suggests that the liquefaction produced by the acetate salt may be due, in part at least, to the accumulation of K.

Discussion

Evidence has been presented that the salts of penetrating acids are able to enter sea urchin eggs within four minutes or less, although certain other ionized compounds are apparently not able to penetrate these cells. The evidence is based on the observation that the salt of a penetrating acid alters the degree to which the acid is able to affect the apparent viscosity of the protoplasm, an effect presumably exerted by virtue of the influence of the acid-salt mixture on the intracellular hydrogen ion equilibria.

This evidence of the penetration of cells by the salts of penetrating acids supports the conclusions arrived at by Smith with other methods on marine ova and cardiac muscle, and by Haywood on skeletal muscle, so that this type of permeability seems to be found in a certain variety of cells. These findings differ from those of Jacobs, Lillie, Osterhout, and others on a number of other cells, and it seems reasonable to suppose that the different results may be due to rather considerable differences in the relative rates of penetration of certain acids and their salts into the various types of cells in question. Since, however, it was noted by Jacobs that the differential effects produced by CO_2–bicarbonate mixtures on *Symphytum* and on starfish eggs tended gradually to disappear, the difference is probably only a quantitative one. This view is substantiated further by the observation of M. M. Brooks (1923) that bicarbonate penetrates into *Valonia* after eighty minutes' exposure. Lillie's observations on starfish eggs were limited to exposures of five to fifteen minutes, and under these conditions the pH of the medium usually seemed to be a less important factor physiologically than the changes in the concentration of acid available for diffusion into the cell; however, the experiments do not exclude the possibility of the penetration of salt.

The present observations of the effects of acids on protoplasmic

viscosity have been discussed in terms of permeability. It has been shown by other authors that many organic acids diffuse through certain living tissues where mineral acids do not. Since organic acids applied externally cause color changes in granules stained by indicators in marine ova although mineral acids do not have this effect, it is probable that the fatty acids actually penetrate. If fatty acid enters marine ova, it is assumed, until proven otherwise, that the salt penetrates and buffers the acid directly in the cell, rather than that it acts from a distance in some unknown manner. Strictly, the term penetration has only the justification of being a convenient description of the selective physiological reactivity with which the present study is concerned.

Summary

The effects of the pH of the medium on the apparent viscosity of *Arbacia* eggs have been studied by means of an adaptation of the centrifuge method.

It was found that the degree to which fatty acids decrease the protoplasmic viscosity can be altered by the presence of the salt of the acid, apparently by virtue of the influence of the salt on the intracellular hydrogen ion equilibria. Similar pH variations of the medium produced by mineral acids do not affect viscosity.

These results offer confirmation, from a separate type of evidence, of Smith's observation that the salts of penetrating acids differ from other ionized compounds in apparently being able to penetrate the living cell much more easily. It is suggested that cell permeability to this type of salt could be explained as resulting from a cation exchange of external base for internal hydrogen ions from the penetrating acid, the entrance of the salt therefore being accomplished without the direct transfer of its anions.

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